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The murine Fgfrl1 receptor is essential for the development of the metanephric kidney

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ABSTRACT

Fgfrl1 is a novel member of the fibroblast growth factor receptor family. Its extracellular domain resembles the four conventional Fgfrs, while its intracellular domain lacks the tyrosine kinase domain necessary for Fgf mediated signal transduction. During embryonic development *Fgfrl1* is expressed in the musculoskeletal system, in the lung, the pancreas and the metanephric kidney. Targeted disruption of the *Fgfrl1* gene leads to the perinatal death of the mice due to a hypoplastic diaphragm, which is unable to inflate the lungs. Here we show that *Fgfrl1*-/- embryos also fail to develop the metanephric kidney. While the rest of the urogenital system, including bladder, ureter and sexual organs, develops normally, a dramatic reduction of ureteric branching morphogenesis and a lack of mesenchymal-to-epithelial transition in the nephrogenic mesenchyme result in severe renal dysgenesis. The failure of nephron induction might be explained by the absence of the tubulogenic markers Wnt4, Fgf8, Pax8 and Lim1 at E12.5 of the mutant animals. We also observed a loss of Pax2 positive nephron precursor cells and an increase of apoptosis in the cortical zone of the remnant kidney. *Fgfrl1* is therefore essential for mesenchymal differentiation in the early steps of nephrogenesis.

Introduction

The mammalian kidney, or metanephros, develops through a complex series of mesenchymal–epithelial interactions when the ureteric bud, an outgrowth of the nephric duct, is induced and invades the metanephric mesenchyme on the level of the hindlimb bud. During this invasion process the epithelial ureteric bud undergoes a series of branching events, ultimately forming the collecting duct system of the adult kidney, which is responsible for urine flow to the bladder. Branching of the ureteric bud and formation of invasive ureteric tips is induced and driven by signals from the metanephric mesenchyme (Grobstein, 1956; Saxen and Sariola, 1987). The ureteric tips in turn induce the proliferation, condensation and ultimately the conversion of adjacent mesenchymal cells into epithelial nephron progenitors (Mori et al., 2003). Although this reciprocal inductive system has been extensively studied and is now well described, relatively little is known about the molecular events that take place during the conversion of mesenchymal cells into the earliest nephron progenitor, the renal vesicle. Mesenchymal cells in proximity to the ureter tips proliferate and condense to form the cap mesenchyme structure. A subset of the cap cells differentiates into the pretubular aggregate (Sariola, 2002) which then undergoes a mesenchymal to epithelial transition to form a polarized epithelial vesicle. This renal vesicle further differentiates into the comma-shaped body, followed by the S-shaped body and finally matures into nephron epithelium (Costantini, 2006; Dressler, 2006). The Wnt family of growth factors is believed to play a key instructive role in this process, with Wnt9b and Wnt11 being secreted from the ureteric bud into the adjacent mesenchyme (Majumdar et al., 2003; Schmidt-Ott and Barasch, 2008) and Wnt4 being synthesized by the condensing mesenchymal cells themselves (Schedl, 2007). Indeed, genetic ablation of *Wnt4* and *Wnt9b* in mice leads to a failure of mesenchymal induction and consequently to the absence of differentiated nephrons (Carroll et al., 2005; Stark et al., 1994). In this context, Wnt9b acts upstream of Wnt4, as missing inductive Wnt9b signaling from the ureter tips results in an absence of *Wnt4* expression in the mesenchymal aggregates (Carroll et al., 2005). Based on in vitro cell induction assays (Kispert et al., 1998; Stark et al., 1994) and mouse mutant studies *Wnt4* itself is necessary and sufficient to drive the transition from aggregated mesenchymal cells into the epithelium of the polarized renal vesicles (Stark et al., 1994).

The fibroblast growth factors and their receptors represent another signaling system that is required for the early steps of nephrogenesis both in the ureter and in the metanephric mesenchyme. Various Fgf ligands, among them Fgf2, Fgf7, Fgf8 and Fgf10, are

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produced in mesenchymal and ureteric compartments of the developing kidney (Barasch et al., 1997; Grieshammer et al., 2005; Qiao et al., 1999; Ohuchi et al., 2000), whereby Fgf7 and Fgf10 signal through Fgfr2IIIb in the ureteric epithelium, while Fgf2 and Fgf8 bind to and activate Fgfr1 and Fgfr2 in the metanephric mesenchyme (Bates, 2007). Fgf7 and Fgf10 as well as Fgfr1 and Fgfr2 seem to have overlapping and partly redundant roles as single mutant mice display only mild kidney defects (Bates, 2007). However, both expression of a soluble, dominant negative *Fgfr* (Celli et al., 1998) as well as double, tissue specific deletions of *Fgfr1* and *Fgfr2* in the metanephric mesenchyme (Poladia et al., 2006) led to renal aplasia, highlighting the crucial importance of Fgfr signaling in kidney development (Bates, 2007). Conditional deletion of *Fgf8* from the metanephric mesenchyme also results in severe renal defects due to an arrest of nephron differentiation at the renal vesicle stage. This arrest is characterized by an absence of *Wnt4* and *Lim1* expression and by an increase of apoptosis in the cortical zone (Grieshammer et al., 2005; Perantoni et al., 2005).

In this study we address the function of a novel member of the Fgfr family, the fibroblast growth factor receptor-like 1 (Fgfrl1) in the development of the metanephric kidney. Fgfrl1 closely resembles the four conventional Fgfrs, as its ectodomain has three extracellular Ig-like domains sharing up to 50% sequence similarity with the other receptors (Wiedemann and Trueb, 2000; 2001). Its intracellular domain, however, comprises only about 100 amino acids and lacks the tyrosine kinase domain that would be required for Fgf ligand mediated autophosphorylation and signal transduction (Sleeman et al., 2001; Trueb et al., 2003). During embryonic development *Fgfrl1* is expressed in cartilage, bone, muscles of the diaphragm and tongue, in the pancreas and in the lung (Kim et al., 2001; Trueb and Taeschler, 2006). In the developing kidney *Fgfrl1* expression is upregulated in the renal vesicles of the differentiating metanephric mesenchyme (Brunskill et al., 2008; www.genepaint.org). Mice with a targeted disruption of the *Fgfrl1* gene (Baertschi et al., 2007) die immediately after birth due to a hypoplastic diaphragm, which renders the newborn animals unable to inflate the lungs. In addition there are subtle alterations in the bones of the skull and in the long bones of the limbs. The most striking phenotype in the otherwise normally developed mice, however, is the fully penetrant severe dysgenesis of both metanephric kidneys. In this study we present evidence that this renal dysgenesis in *Fgfrl*-/- mice is caused by a failure of metanephric mesenchymal cells to differentiate into nephron epithelium accompanied by severe branching defects of the ureter.

Materials and methods

Transgenic animals

Fgfrl1-/- mice were generated and bred as previously described (Baertschi et al., 2007). Littermates were used for all comparative experiments between wild-type and transgenic embryos. For an exactly timed pregnancy, the noon of the day, on which a vaginal plug was detected, was considered as E0.5.

Histology and immunohistochemistry

Embryos of different developmental stages were dissected, fixed in 4% paraformaldehyde (PFA) and cut into 5-μm sections. The sections were stained with Mayer's hemalaun and eosin (H&E). For immunohistochemistry, the sections were boiled in citrate buffer to unmask the antigens, washed in Tris buffered saline (TBS) containing 0.025% Triton X-100 and blocked with 3% bovine serum albumin (BSA) in TBS. The following primary antibodies were used: anti-pan cytokeratin monoclonal antibody PCK-26 (1:600, Sigma), anti-calbindin D28K monoclonal antibody CB-955 (1:100, Sigma), anti-laminin polyclonal antibody 143 (1:200, a kind gift from Dr. M. Chiquet, University of Bern), anti-Pax2 polyclonal antibody PRB-276P (1:100, Covance, Emeryville, CA), and anti-Wt1 polyclonal antibody C-19 (1:100, Santa Cruz Biotechnology Inc.). After incubation overnight with the primary antibody at 4 °C, the following secondary antibodies were applied: TRITC conjugated goat anti-rabbit antibodies (1:400; Sigma) and Cy2 conjugated goat anti-mouse antibodies (1:100; Jackson Immunoresearch Laboratories). For detection of Fgfrl1, the polyclonal goat antibodies FGFR5β (1:100; R&D Systems) and FGFR5 C-20 (1:100; Santa Cruz Biotechnology Inc.) were used. To detect the bound antibodies, secondary rabbit anti-goat antibodies conjugated with alkaline phosphatase were employed (1:150, Sigma). The color reaction was performed with bromochloroindolyl phosphate and nitroblue tetrazolium substrate.

Metanephric organ cultures

Kidney rudiments were dissected from E11.5 embryos and cultured for up to 96 h on polycarbonate transwell filters (0.4 μm, Falcon 3180) at the liquid–gas interphase (Saxen and Lehtonen, 1987). Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, non-essential amino acids and penicillin/streptomycin served as culture medium. For immunofluorescence experiments, the organ cultures were excised together with the transwell filters, fixed for 30 min with 4% PFA in PBS, permeabilized for 1 h with 1% Triton X-100 in PBS and blocked with 3% BSA in PBS. After incubation overnight at 4 °C with primary antibodies, the cultures were washed for a total of 10 h with several changes of 0.1% Triton X-100 in PBS. Fluorescently labeled secondary antibodies were added and incubation was repeated overnight. After extensive washing as above, the cultures were mounted with Mowiol on glass slides and photographed under a Nikon Eclipse E1000 microscope.

Proliferation and apoptosis assays

Selected tissues of E14.5 littermates were fixed in 4% PFA and embedded in Tissue-Tek OCT Compound (Sakura Fintek, Zoeterwoude, the Netherlands). The frozen tissues were sectioned at 7 μm and fixed again in PFA. After permeabilization with 0.1% Triton X-100 in PBS, the sections were blocked with 3% BSA in PBS. Apoptotic cells were detected by TUNEL utilizing the In situ Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. Cell proliferation was determined in whole-mount organ cultures with the BrdU Labeling and Detection Kit I (Roche Diagnostics). For this purpose, the organ cultures were incubated at 37 °C with BrdU containing labeling medium (1:1000). After 2 h the specimens were fixed with 70% ethanol, permeabilized with 1% Triton X-100, blocked with 3% BSA and incubated with a mouse antibody against BrdU (1:10, Roche Diagnostics) as well as with a rabbit antibody against laminin (see above). After extensive washing, the corresponding secondary antibodies were added, a fluorescein conjugated sheep anti-mouse Ig (1:10, Roche Diagnostics) and the Cy3 conjugated goat anti-rabbit Ig (see above).

Quantitative PCR

Total RNA was extracted from pooled kidney rudiments ($n = 6–9$) of mutant and wild-type embryos using the RNeasy kit from Qiagen GmbH (Hilden, Germany). The RNA was transcribed into first strand cDNA by reverse transcriptase from Moloney Murine Leukemia Virus (MMLV, Stratagene). Primer pairs for selected gene products (Table S1) were designed with the Probe Library Tool from Roche Diagnostics and ordered from Microsynth AG (Balgach, Switzerland). The cDNAs were quantified by real time PCR utilizing the ABI 7500 platform (Applied Biosystems).

Whole-mount in situ hybridization

In order to generate hybridization probes for various genes, cDNA from E16.5 kidneys was amplified by PCR with the primer pairs listed in Table S2 and the products were subcloned into the expression vector pSPT19 (Roche). Riboprobes were transcribed from the linearized vectors in the presence of digoxigenin-labeled UTP using the DIG RNA Labeling Kit (SP6/T7) (Roche).

Whole-mount in situ hybridization was carried out by established procedures (Piette et al., 2008; Wilkinson and Nieto, 1993) with modifications described on the GUDMAP gene expression database (http://www.gudmap.org/Research/Protocols/ McMahon.html). Briefly, kidneys from 12.5- and 14.5-day-old embryos were dissected and fixed overnight in 4% PFA. The kidneys were dehydrated through a graded methanol series (25%, 50%, 75%, 100%) diluted in PBS containing 0.1% Tween 20 and then stored at -20 °C. The samples were rehydrated, bleached for 30 min with 6% hydrogen peroxide and digested for 15 min with Proteinase K (10 μg/ml). The tissues were refixed in 0.2% glutaraldehyde/4% PFA, prehybridized for 2 h at 68 °C in hybridization solution (50% formamide, 5X SSC (saline-sodium citrate), 1% SDS, 50 μg/ml yeast t-RNA, 50 μg/ml heparin) and incubated overnight at 68 °C with the riboprobes. After high stringency washes (50% formamide, 5X SSC, 1% SDS) at 68 °C, the samples were blocked for 2 h at RT with 3% BSA in TBST and incubated overnight at 4 °C with pre-absorbed anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Roche, 1:2000). Following extensive washes, BM Purple (Roche) was added to develop the hybridization signal. After 12–24 h at RT, the samples were photographed with a Nikon Coolpix 990 digital camera.

Results

Fgfrl1 is expressed in nascent nephrons

In order to investigate the effects of *Fgfrl1* deficiency on kidney development, we analyzed the expression of *Fgfrl1* in embryonic kidneys from wild-type animals (Fig. 1). Microarray analysis and in situ hybridization of Fgfrl1 mRNA had previously shown that this receptor is expressed in the metanephric mesenchyme, with strongest expression observed in renal vesicles and nascent nephrogenic structures (Brunskill et al., 2008; www.gudmap.org; www.genepaint.org). Our immunostaining experiments with a polyclonal antibody against the extracellular domain of Fgfrl1 (Fig. 1A) confirmed these data. The highest signal was detected in differentiating renal vesicles and in the nephrogenic structures derived thereof. Weaker staining was seen in the undifferentiated mesenchymal cells and in the ureteric epithelium. No expression was detected in the stromal cells surrounding the nephrogenic structures. The larger magnifications provided in Fig. 1A demonstrate that Fgfrl1 protein was enriched at sites of cell–cell contact, especially in the nascent epithelial bodies of renal vesicles and further developed nephron precursors. The expression pattern suggests that *Fgfrl1* could be involved in the differentiation of mesenchymal nephron precursor cells into tubular epithelial structures. Quantification of *Fgfrl1* expression by real time PCR was in good agreement with this observation (Fig. 1B). During embryonic development of wild-type kidneys, the mRNA level for *Fgfrl1* increased from E11.5 to E12.5, probably reflecting the onset of nephrogenesis. It reached a maximum around E16.5 and decreased sharply thereafter when nephrogenesis eventually ceases.

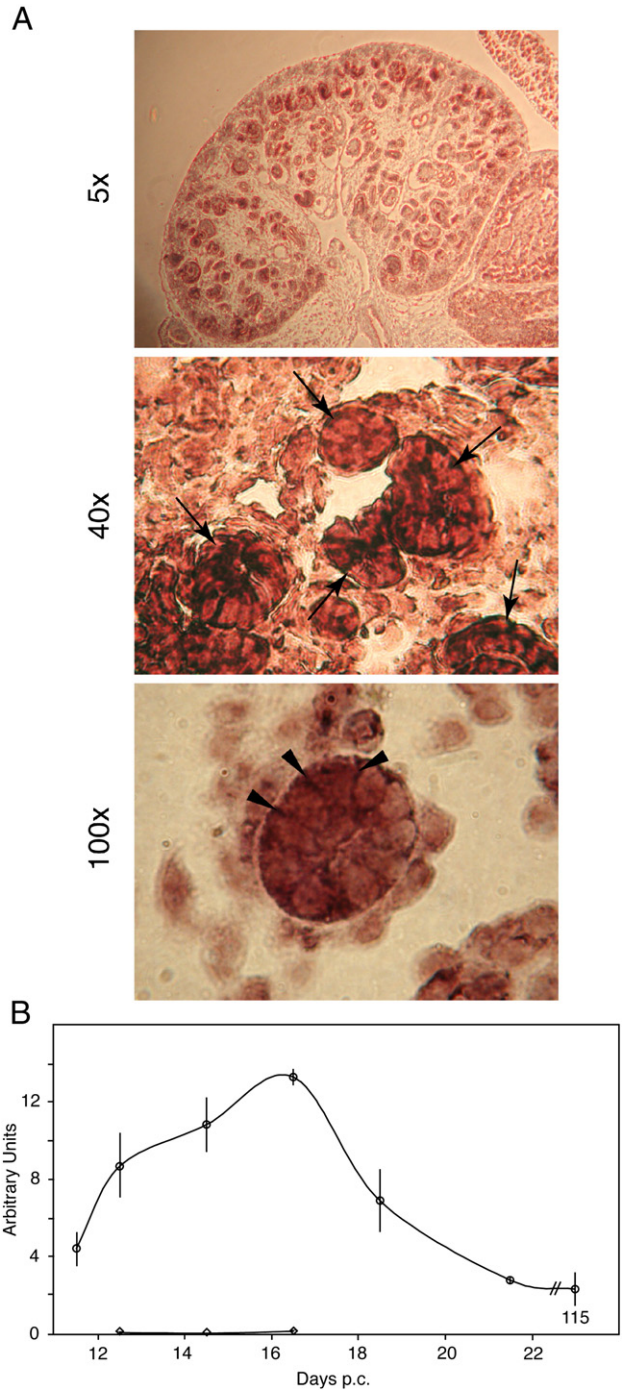


Fig. 1. Expression of *Fgfrl1* in wild-type E15.5 kidneys. (A) Immunohistochemistry with a polyclonal antibody against the Fgfrl1 ectodomain reveals Fgfrl1 protein in nephrogenic structures of the cortical zone, with strongest signal observed in renal vesicles, comma and S-shaped bodies. The cortical and medullary stroma and the fully differentiated glomeruli display no Fgfrl1 protein. The 40× magnification shows an area from the cortical nephrogenic zone. Nascent nephrons ranging from renal vesicles to S-shaped bodies are indicated by black arrows. The nascent nephrons contain Fgfrl1, but the surrounding cells do not. The 100× magnification shows a renal vesicle from the cortical zone. Fgfrl1 protein is enriched at sites of cell–cell contact in the newly formed epithelium of the renal vesicle (arrow heads). (B) Quantification of the *Fgfrl1* mRNA in the developing kidneys. *Fgfrl1* expression is maximal around E16 in the wild-type kidneys (l) ●. Before E12 and after birth, expression is very low. Kidney rudiments of *Fgfrl1* deficient mice (◆) do not express *Fgfrl1*.

Severe bilateral renal dysgenesis

Heterozygous *Fgfrl1*+/- mice in a C57BL/6 background displayed no overt phenotype (Baertschi et al., 2007). They were fertile and produced offspring in the expected Mendelian ratio. Although our *Fgfrl1*-/- embryos were born alive and appeared to be normally developed, they died immediately after birth due to a hypoplastic diaphragm muscle, which was unable

to inflate the lungs. When we systematically screened our *Fgfrl1*-/- embryos for additional organ defects, we discovered that the metanephric kidneys were missing in all of the examined animals (Fig. 2). At embryonic day E18.5, only a small, rudimentary structure that was attached to the ureter was found in place of the kidney. The rest of the urogenital system, including testes, ovaries, bladder and ureter, appeared to be normally developed.

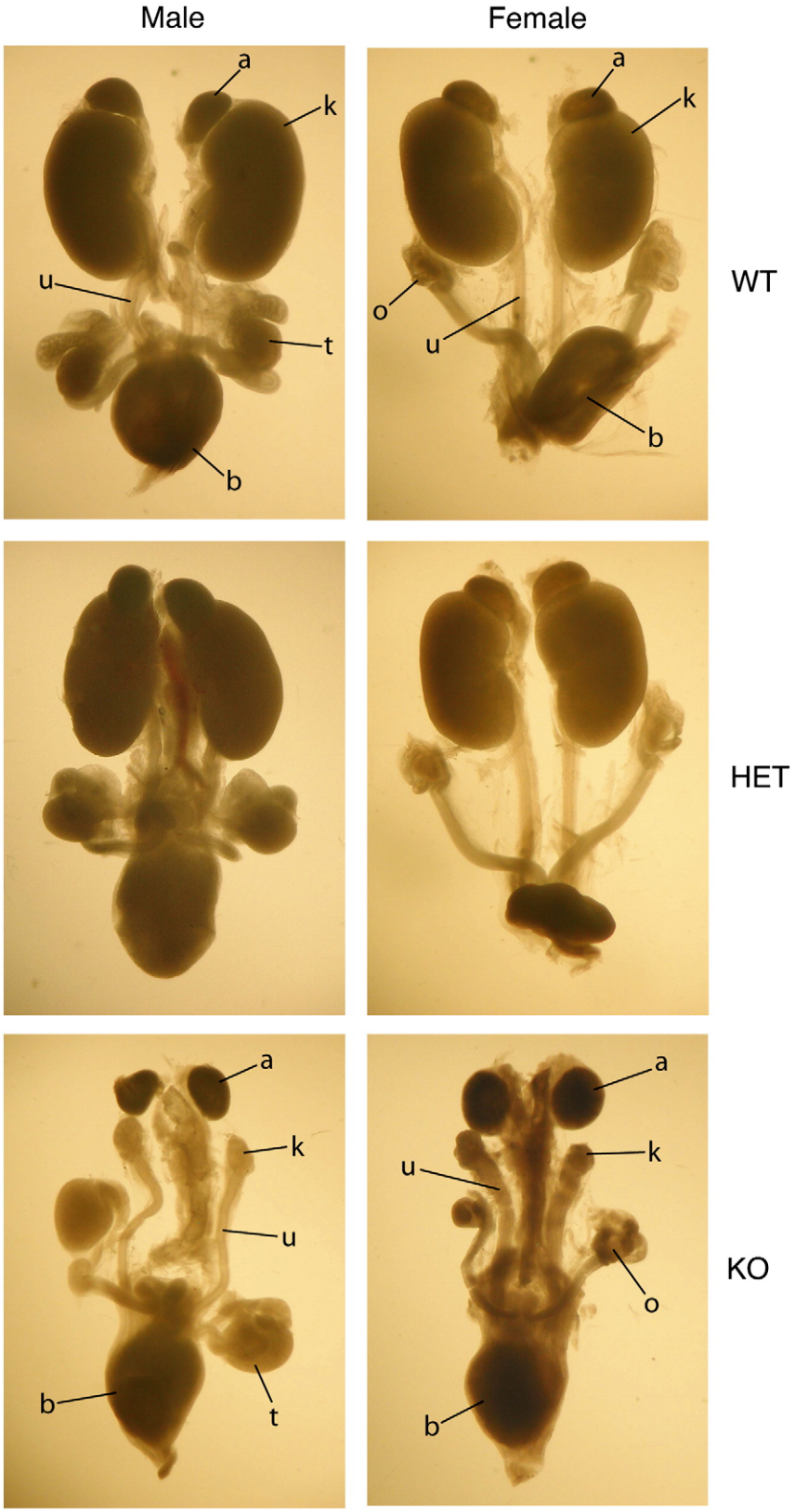
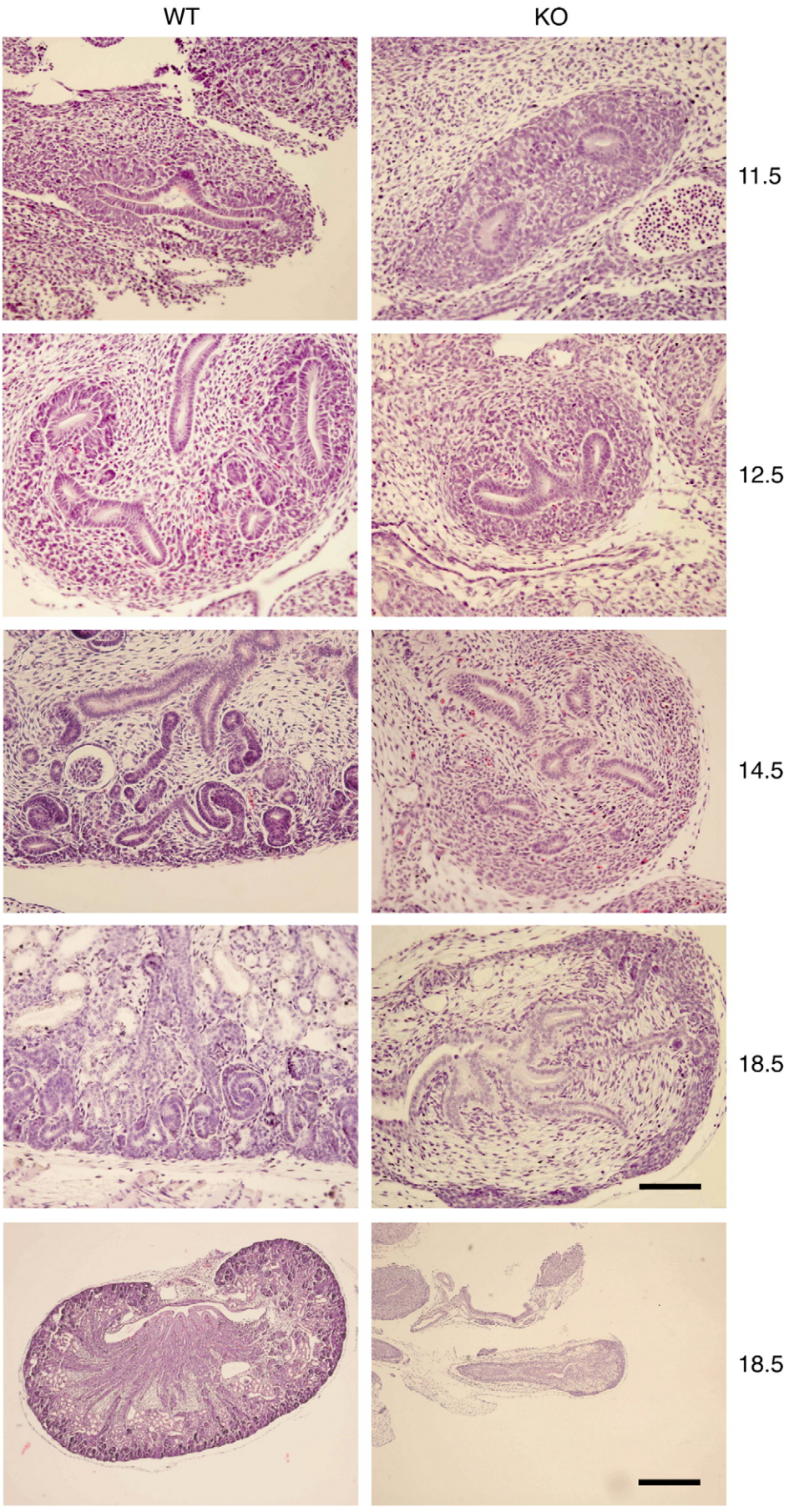


Fig. 2. Severe renal dysgenesis in *Fgfrl1*-/- mice. The urogenital system of wild-type (WT), heterozygous (HET) and knock-out (KO) embryos is shown at E18.5. The mutant mice exhibit only rudimentary structures in place of the kidneys. Ureter, bladder and female and male gonads appear to be normally developed. a, adrenal gland; b, bladder; k, kidney; o, ovary; t, testis; u, ureter.


WT
KO
11.5
12.5
14.5
18.5
18.5

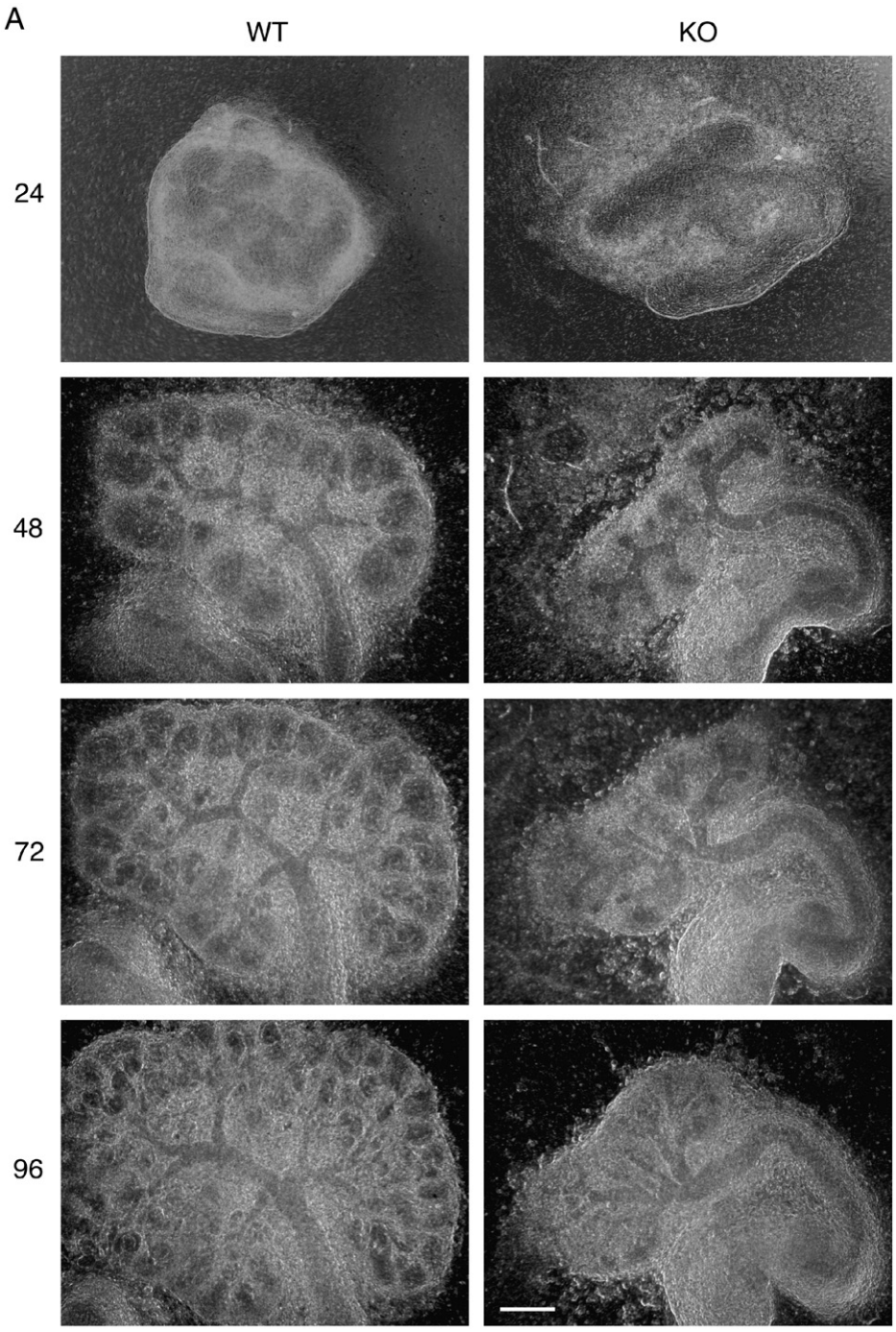


Fig. 4. Whole kidney organ cultures recapitulate the developmental defects in *Fgfrl1* deficient mice. (A) Kidney rudiments of E11.5 wild-type and mutant littermates were dissected and cultured on transwell filters for 96 h. The wild-type cultures develop normally with a symmetrical branching pattern and visible differentiation of the mesenchyme. The mutant cultures show severely reduced and uncoordinated ureteric branching and a lack of tubulogenesis. Bar = 200 μm. (B) Whole-mount immunofluorescence with the ureteric bud marker calbindin. Three representative examples are shown. (C) Statistical analysis of tip formation and (D) ureteric branching events after three days in culture. The wild-type ureteric buds branch six to seven times, resulting in 55 tips ($n = 7$). In contrast, the knock-out ureters branch only three to four times, resulting in 20 tips on the average ($n = 8$).

Defects in ureteric branching and mesenchymal differentiation

In order to investigate the time point and nature of the developmental arrest of the kidney, sections containing the kidney anlagen from E11.5 to E18.5 embryos were examined after H&E staining (Fig. 3). At E11.5 the ureteric bud of wild-type and mutant animals had begun to invade the metanephric mesenchyme and branched once to form the so called "T-stage." At E12.5 the ureter had undergone another round of branching and the adjacent mesenchyme had condensed around the ureter tips to form the cap mesenchyme. At

Fig. 3. Severe defects in branching and mesenchymal differentiation. H&E staining of kidney sections from E11.5 to E18.5 reveals that branching of the *Fgfrl1*-/- ureter and mesenchymal differentiation are severely reduced. T-stage of the mutant ureteric bud and metanephric mesenchyme appear to be normal at E11.5. At E12.5 the ureteric bud of the knock-out animals is still in the T-stage and tubulogenesis is not initiated. From E12.5 to E18.5 nephrogenesis and ureteric branching proceed in the wild-type kidneys, while no nephrons form in the mutant kidneys. Morphogenesis of the ureteric bud and collecting duct system is arrested after a few rounds of branching in the mutants. The scale bar is equal to 100 μm for all panels except the two panels at the bottom where it is equal to 500 μm.

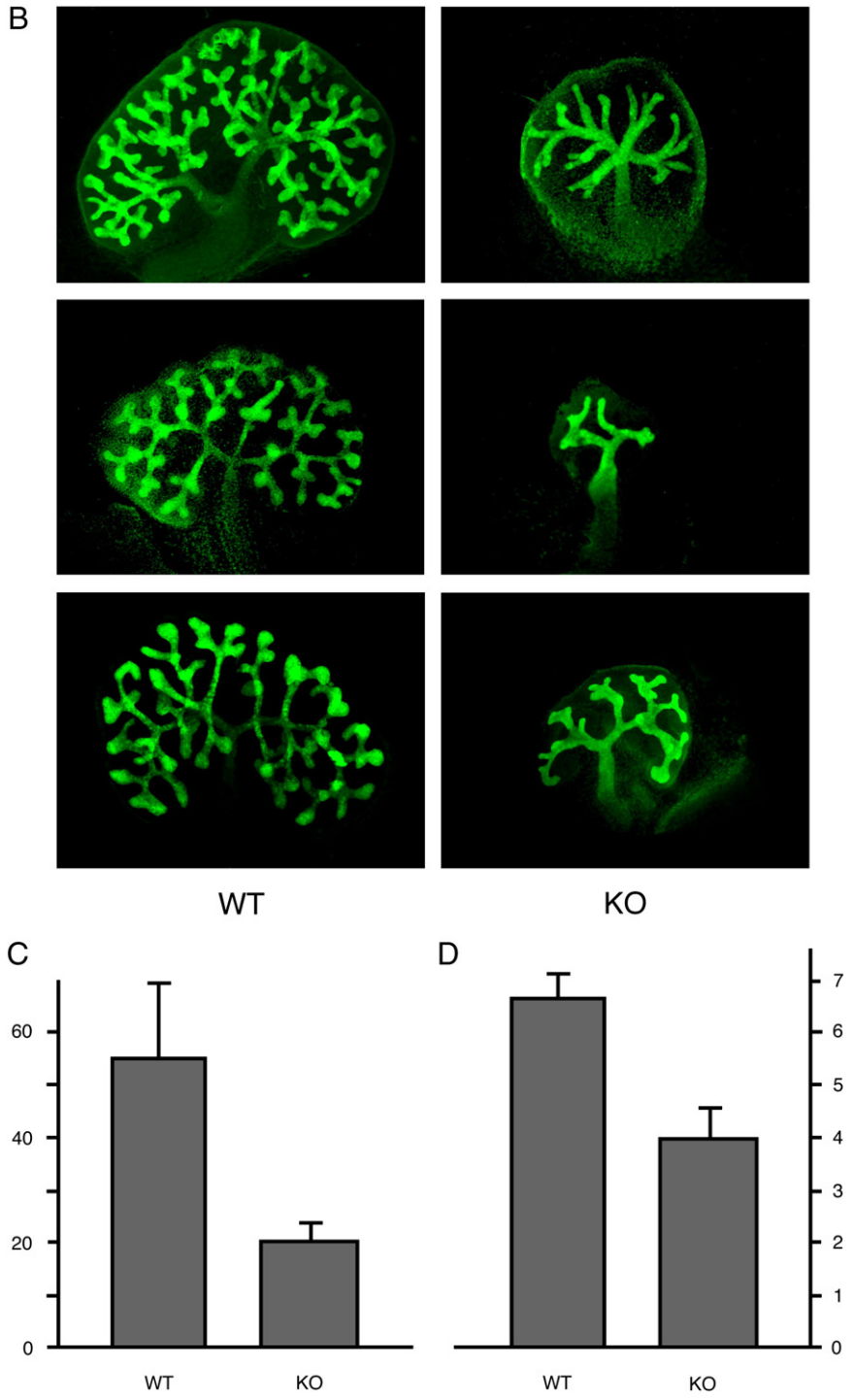


Fig. 4 (*continued*).

this stage the first epithelial renal vesicles appeared in the wild-type animals. In the *Fgfrl1*-/- embryos less mesenchymal condensation was seen at this stage and renal vesicles were absent. The differences became more striking at E14.5 when the wild-type ureter had branched multiple times and the first nephrons had formed. At this stage branching of the mutant ureter appeared to have stalled and no epithelial structures had differentiated from the metanephric mesenchyme. At E18.5 the kidney was almost fully developed in the wild-type animals, whereas no nephrons had formed in the mutant animals and the ureteric collecting duct system showed severely reduced branching. The absence of nephrons and the malformed ureteric collecting duct system indicated that *Fgfrl1*-/- mice lack functional metanephric kidneys.

In addition to these in vivo studies we made use of the well established whole kidney organ culture system (Saxen and Sariola, 1987; Saxen and Lehtonen, 1987) to gain insight into the effects of *Fgfrl1* deficiency on kidney development. Kidney rudiments of wild-type and mutant animals were dissected at E11.5 and cultured on polycarbonate filter membranes. Fig. 4A shows a comparison of wild-type and mutant kidney cultures grown over a time period of 96 hours. The wild-type cultures developed normally with a rapidly and symmetrically branching ureteric bud and clear induction of nephrogenic structures in the

mesenchyme. In sharp contrast, the mutant cultures showed aberrant and severely reduced branching together with little or no visible differentiation of the mesenchyme. Branching of the mutant ureter appeared irregular and less coordinated than that of the wild-type ureter and mesenchymal condensation, pretubular aggregation and tubulogenesis were greatly reduced or virtually absent.

Whole-mount immunofluorescent staining of the kidney cultures with the ureteric epithelium marker calbindin allowed for a statistical analysis of ureteric tips and branching events in wild-type and mutant cultures after 72 h (Figs. 4B–D). While the wild-type ureters had branched six to seven times forming about 55 tips, the *Fgfrl1*-/- ureters had branched only three to four times resulting in an average of 20 tips. The overall size of the mutant kidney cultures was greatly reduced when compared to the cultures of wild-type littermates.

In combination, the in vivo studies and the organ cultures suggest that the ureteric bud of *Fgfrl1*-/- embryos is initially induced by the metanephric mesenchyme. It is able to undergo some rounds of branching and tip formation. In this context it should be noted that the ureteric bud undergoes more branching events in the organ cultures than in the living animals. However, tubulogenesis of the mesenchyme does not appear to be initiated at all, neither in the organ cultures nor in vivo.

Absence of nephrogenic markers in the Fgfrl1-/- mesenchyme

The histological analysis of kidney development in the mutant mice suggested a severe defect in branching morphogenesis and mesenchymal differentiation that became apparent at E12.5. We therefore extracted RNA from E12.5 and E14.5 kidney rudiments and performed real-time RT-PCR to assess the expression of marker genes known to be important for the early steps of nephrogenesis. Table 1 lists the full set of genes analyzed and the changes in the expression levels between wild-type and mutant kidney rudiments. Although many of the genes were slightly down-regulated in the mutant kidney rudiments, we found a number of genes that showed a striking reduction in their expression levels. The most pronounced reduction was observed for the transcription factors *Pax8* and *Lim1* as well as for the secreted factors *Wnt4* and *Fgf8*, all of which are marker genes of the tubulogenic program (Carroll et al., 2005). *Pax2* expression, which occurs both in the uninduced mesenchyme and in the developing nephrons (Narlis et al., 2007) was also significantly reduced, albeit not to the same extent as *Pax8*. The expression of *Eya1*, *Six2*, and *Wt1*, which, together with *Pax2*, define the uninduced mesenchyme (Brodbeck and Englert, 2004), was largely intact. Likewise, the ureteric genes *Wnt9b*, *Wnt11* and *c-Ret* were still expressed at E12.5. These expression data point to a defect in tubulogenesis due to failure of the mesenchyme to express tubulogenic genes.

Table 1
Genes showing altered expression in kidneys of Fgfrl1 knock-out embryos.

Gene	Fold change (wt/ko)	
	E12.5	E14.5
Wnt4	13.3	4.2
Pax8	9.6	7.1
Fgf8	9.2	12.1
Lim/Lhx1	7.7	18.3
Fgfrl1	6.8	21.2
Pax2	5.4	4.1
Eya1	3.1	5.4
Ret	3.1	8.9
Gdnf	3.0	3.4
Osr1	2.8	2.1
Emx2	2.4	5.2
Fgfr1	2.1	1.3
Wnt11	2.0	2.0
Wt1	2.0	3.1
Wnt9b	1.9	1.7
Six1	1.6	1.5
Fgfr2	1.5	1.3
Six2	0.7	4.3
Ehf	0.3	0.7
Gapdh	1.0	1.0

We next set out to characterize the mutant mesenchyme and the apparent lack of nephrogenesis in detail. To this end we performed immunohistochemistry with antibodies against the mesenchymal markers Pax2 and Wt1 on kidney sections and in whole organ cultures. We also analyzed the expression of the ureteric genes *Wnt9b* and *Wnt11* and of the tubulogenic markers *Pax8*, *Fgf8*, *Wnt4* and *Lim1* by whole-mount in situ hybridization of E12.5 and E14.5 kidneys.

Pax2 is expressed during the entire development of the metanephros, both in the ureteric collecting duct system and in the mesenchymal compartment. In the renal vesicle, *Pax2* and *Pax8* act in concert to drive differentiation from nephron progenitors of the renal vesicle stage to the end of nephron development. *Pax2* homozygous mutant mice form the pronephros and the mesonephros but fail to induce the metanephros (Narlis et al., 2007). As shown by immunohistochemisty (Fig. 5), Pax2 was normally produced at E12.5 and E14.5 in the ureteric compartment of the mutant kidney. However, the mesenchymal compartment showed some reduction in Pax2 synthesis when compared to wild-type kidneys of the same developmental stage. At E12.5 the first renal vesicles and comma-shaped bodies had appeared in the wild-type kidneys and both were found to strongly produce Pax2. The condensing cells of the cap mesenchyme and the pretubular aggregates around the cytokeratin-positive ureteric tips showed high levels of Pax2 protein as well. In contrast, the mutant kidneys completely lacked the epithelial Pax2-positive nephron precursors. Although the mesenchymal cells still synthesized Pax2, only little Pax2-positive condensation and no epithelialization was present around the ureter tips. At E14.5 the differences became even more striking. Numerous Pax2-positive nephron precursors had formed in the wild-type kidneys and Pax2 producing cap mesenchyme surrounded the ureteric tips in the cortical zone, while no nephron differentiation was found in the mutant kidneys. Here, only few isolated cells appeared to synthesize Pax2 and no Pax2-positive cap mesenchyme was observed. The five-fold reduction in *Pax2* expression (Table 1) at E12.5 and E14.5 was most likely due to the absence of the condensing cap mesenchyme and nephron precursor structures, both of which strongly express *Pax2* in the wild-type kidneys.

We also stained the kidney sections for the presence of Wt1 protein (Fig. 5). *Wt1* plays a dual role during kidney development as it is expressed in the metanephric mesenchyme at the earliest stages of nephrogenesis (Donovan et al., 1999) and later on in the podocytes of the glomerulus (Morrison et al., 2008). In the early mesenchyme it specifies, together with *Pax2*, *Eya1* and *Six2* (Brodbeck and Englert, 2004), the nephrogenic precursor cell population, which eventually differentiates into nephron epithelium. Utilizing specific antibodies and sections from E12.5 embryos, we observed that *Wt1* expression was relatively intact in the mutant kidneys, although a lack of condensation and epithelialization was also evident when compared to the wild-type sections. Staining of E14.5 sections from wild-type embryos displayed some Wt1 protein in the cortical mesenchyme and a strong, focal signal in the podocytes of the nascent glomeruli. As expected, no such signal was detected in the sections from the E14.5 mutant animals, providing further evidence for the absence of nephron differentiation (Fig. 5). We also tried to stain the kidney sections with a polyclonal antibody against Six2 but could not obtain any specific signal (not shown). We therefore analyzed its expression by whole-mount in situ hybridization (see below in Fig. 7, bottom). In good agreement with the real-time PCR results, *Six2* expression was intact in the *Fgfrl1* deficient kidneys. Taken together, the *Pax2*, *Wt1* and *Six2* expression analysis suggested that the uninduced metanephric mesenchyme is present at E12.5, but fails to initiate nephrogenesis.

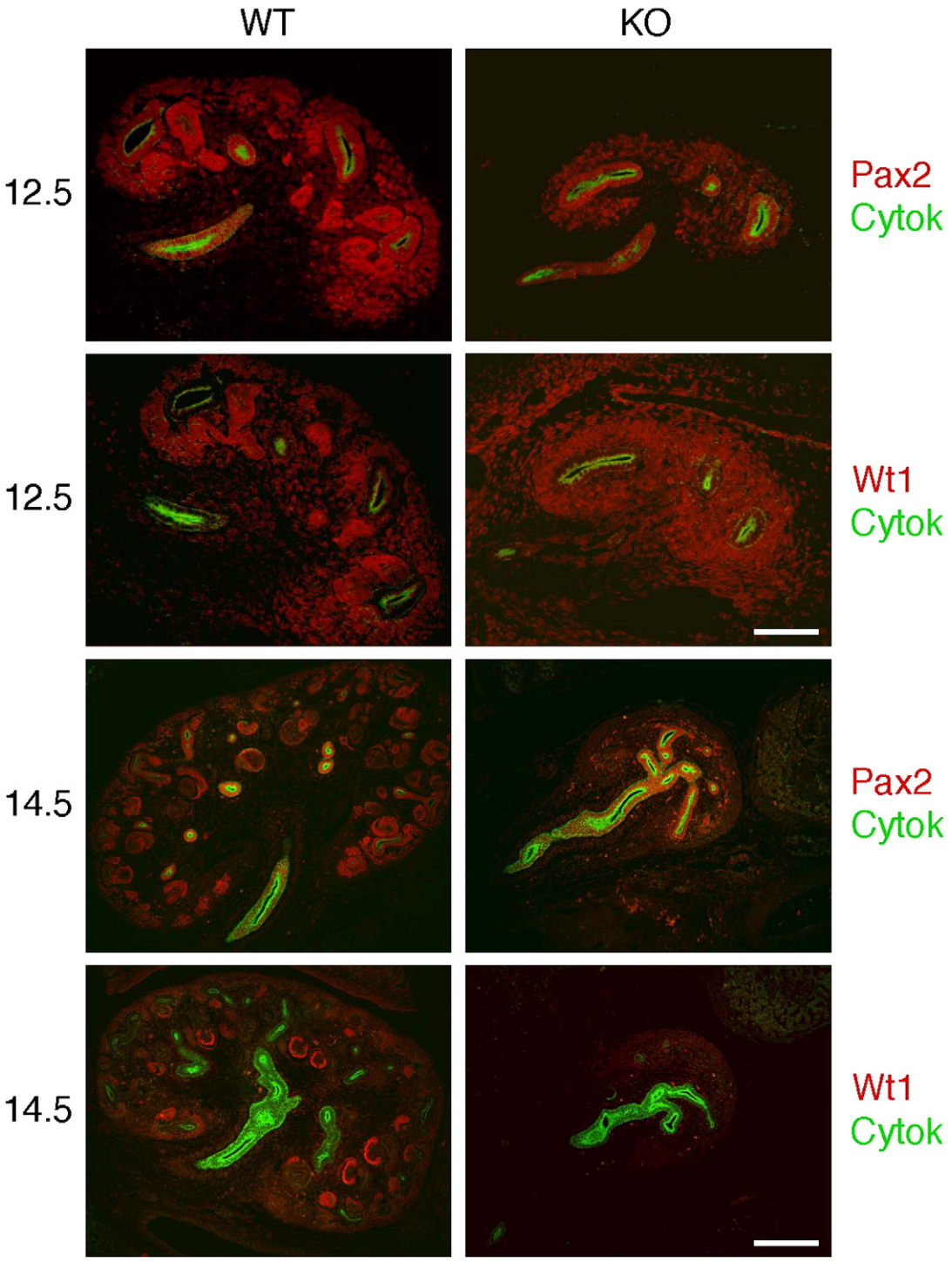


Fig. 5. Analysis of marker gene expression. Paraffin sections from kidneys of *Fgfrl1* wild-type and knock-out littermates of E12.5 and 14.5 were stained with antibodies against cytokeratin, Pax2 and Wt1. In the *Fgfrl1*-/- mice of E12.5, synthesis of Pax2 appears to be normal in the ureteric bud and in the uninduced mesenchymal cells. The first Pax2 positive renal vesicles have formed in the wild-type kidneys, but are missing in the mutants. At E14.5 there is a marked reduction of Pax2 positive mesenchymal cells in the *Fgfrl1*-/- mice and no nephrogenesis has taken place. Wt1, a marker for the uninduced mesenchyme and for podocytes, is still produced in the mutant mesenchyme at E12.5. However, no Wt1-postive podocytes are detected at E14.5 in the mutant kidneys, reflecting a loss of nephron differentiation. Podocytes of the wild-type glomeruli strongly express Wt1 at E14.5. Bar = 100 μm for E12.5 and 200 μm for E14.5.

We also analyzed the expression of *Pax2* and *Wt1* and the lack of tubulogenesis in our kidney cultures. Whole-mount staining of the cultures with antibodies against Pax2 and Wt1 confirmed the results described above (Fig. 6). Expression of *Pax2* appeared to be largely intact in the ureteric bud of the *Fgfrl1*-/- cultures but was found to be reduced in the mesenchymal compartment where no nephrogenesis occurred. This was also reflected by a lack of Wt1-positive podocytes in the mutant cultures, whereas such podocytes were clearly present in the wild-type cultures. Laminin staining of the kidney cultures provided further evidence for the failure of tubulogenesis in the mutant cultures. While an antibody against this marker for basement membranes stained the ureteric epithelium and the newly formed epithelia of the nascent nephrons in the wild-type cultures, it stained only the ureteric bud in the mutant cultures. This suggested that no

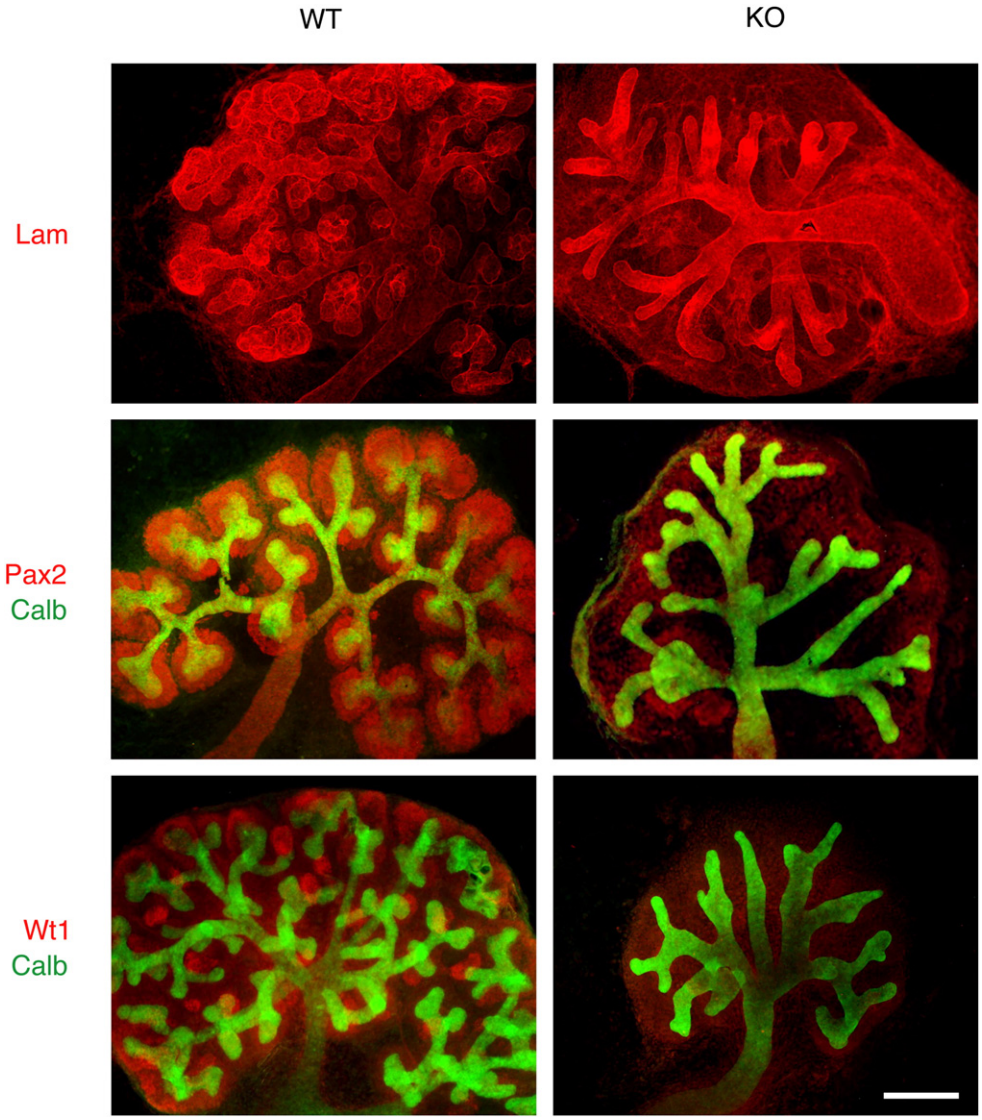


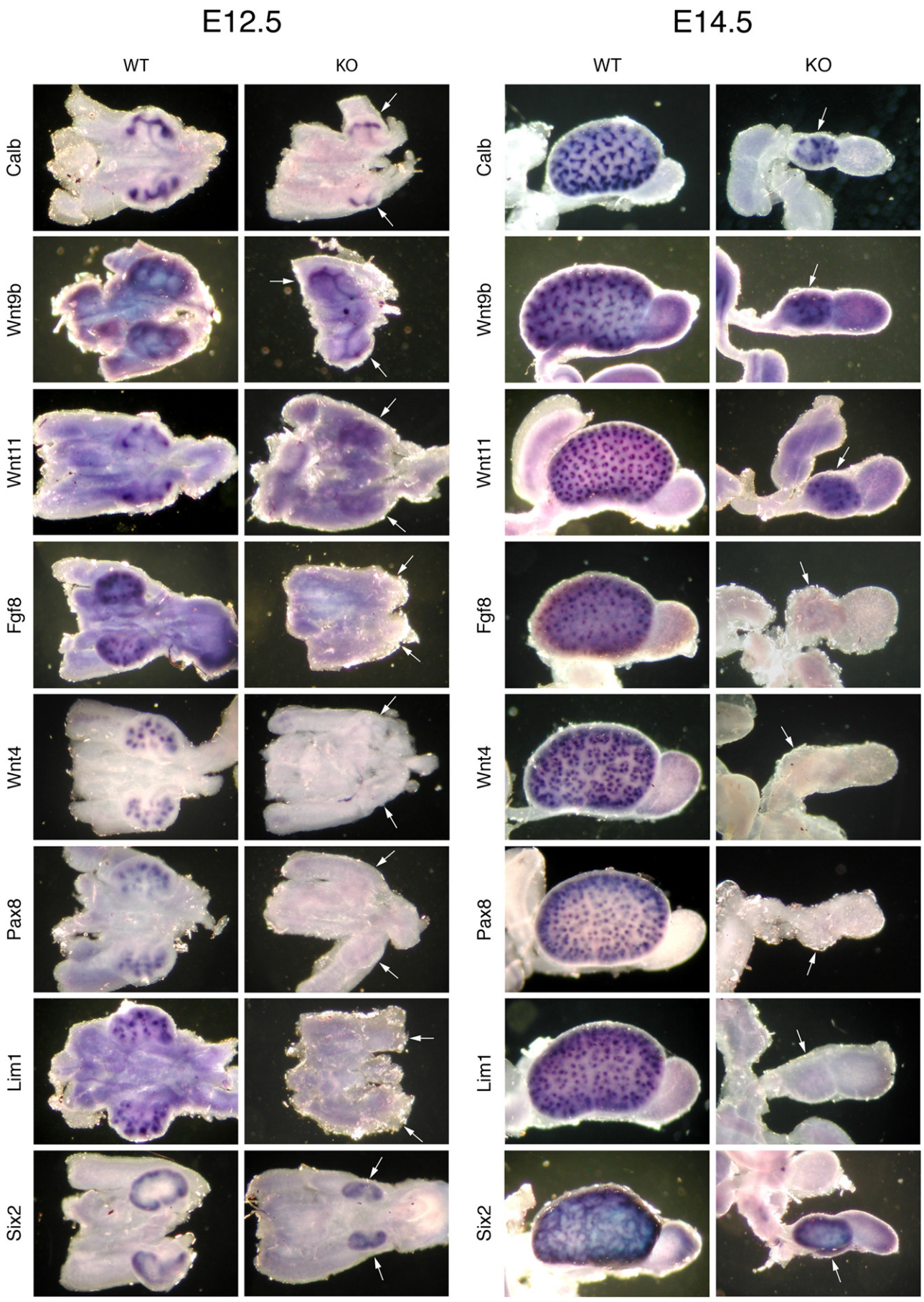
Fig. 6. Whole-mount immunofluorescence of *Fgfrl1*-/- organ cultures confirms the defect of nephron differentiation. Organ cultures from wild-type and knock-out littermates were fixed after 3 days in culture and stained with antibodies against laminin, calbindin, Wt1 and Pax2. The antibodies against laminin stain the epithelium of the ureter and the newly formed nephrons in the wild-type cultures. In the *Fgfrl1* knock-out cultures, only the ureteric epithelium is stained, reflecting the defect in epithelialization of nephron precursors. Pax2 appears to be present in the ureteric bud of the wild-type and knock-out cultures, but the amount of this marker is severely reduced in the mesenchyme. Very few Pax2-positive mesenchymal cells are clustered around the mutant ureteric tips. In the wild-type cultures, Wt1 is produced in the podocytes of the glomeruli. No podocytes are present in the mutant cultures. Bar = 200 μm.

epithelialization of nephrogenic structures took place in the *Fgfrl1*-/- kidney cultures.

The observed defect in nephrogenesis could be due to a loss of inductive signaling from the invading ureter or due to a failure of the mesenchyme to properly respond to those signals. The real-time PCR data suggested that the inductive factors *Wnt9b* and *Wnt11* were still expressed in the ureter, while the mesenchyme remained unresponsive and did not express the tubulogenic markers *Wnt4*, *Fgf8*, *Pax8* and *Lim1*. To validate this, we utilized whole-mount in situ hybridization (ISH) with probes against these gene products. As shown in Fig. 7, the ISH experiments with E12.5 and E14.5 embryos completely confirmed the real-time PCR results. At E12.5 the ureteric bud began to invade the metanephric mesenchyme and expressed *Wnt9b* and *Wnt11* (calbindin was included as a positive control) both in the wild-type and in the mutant embryos, although expression levels in the *Fgfrl1* deficient embryos were reduced to some extent. In response to the inductive signals from the ureter, the wild-type mesenchyme had initiated the tubulogenic program and focally expressed *Wnt4*, *Fgf8*, *Pax8* and *Lim1*. In contrast, none of these genes was expressed in the mutant mesenchyme. At E14.5 there was still no expression of any of the tubulogenic marker genes in the *Fgfrl1* deficient kidneys, while we observed ongoing expression of *Wnt9b* and *Wnt11* by the mutant ureteric stalks and tips, respectively.

Less proliferation and increased apoptosis in Fgfrl1-/- kidneys

It has previously been shown that a deletion of the factors *Fgf8* and *Wnt4* leads to an increase of apoptosis in the developing kidney, especially in the differentiating nephrogenic cortical zone (Grieshammer et al., 2005; Perantoni et al., 2005). We therefore analyzed wild-type and mutant kidney sections of E14.5 for the presence of apoptotic cells by TUNEL (Fig. 8A). In contrast to the wild-type control, which displayed only sporadic cell death with no obvious spatial accumulation, TUNEL staining of the *Fgfrl1*-/- sections revealed a marked increase in the number of apoptotic cells in the peripheral zone of the remnant kidney. The location of the apoptotic cells and their co-staining with the epithelium marker calbindin indicated that the apoptotic cells represented


E12.5
E14.5
WT
KO
WT
KO
Calb
Wnt9b
Wnt11
Fgf8
Wnt4
Pax8
Lim1
Six2
Calb
Wnt9b
Wnt11
Fgf8
Wnt4
Pax8
Lim1
Six2

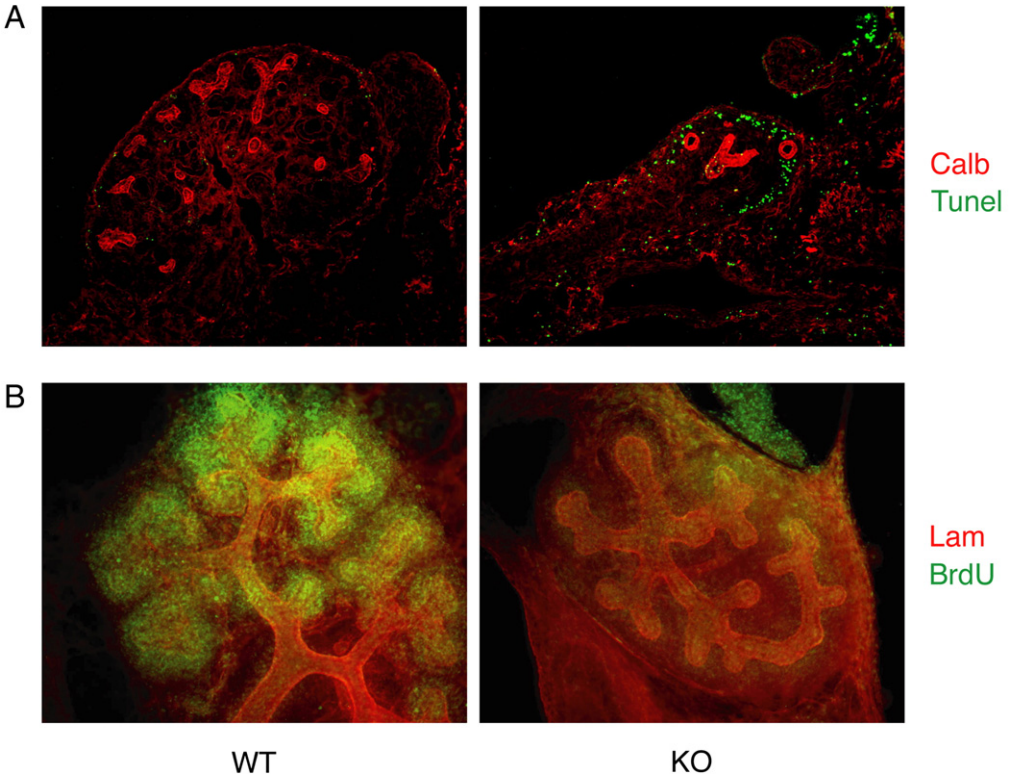


Fig. 8. Increased apoptosis in *Fgfrl1*-/- kidneys and reduced proliferation in mutant organ cultures. (A) TUNEL analysis of apoptotic cell death in cryosections of E14.5 wild-type and mutant kidney rudiments. An increase of apoptosis is detected in the cortical zone of the knock-out sections. (B) Proliferation assay. Organ cultures from wild-type and knock-out kidney rudiments were incubated for 2 h with BrdU and the incorporation was detected with an antibody against BrdU. The dramatically reduced incorporation into the knock-out cultures demonstrates a pronounced lack of proliferation in the *Fgfrl1* deficient mesenchyme.

mesenchymal cells of the cortical nephrogenic zone and not cells of the invading ureter tips.

Using whole organ cultures we further investigated whether, in addition to the increase of mesenchymal apoptosis, the rate of proliferation was altered in the mutant mesenchyme (Fig. 8B). BrdU was added to the cultures and after 2 h the incorporation was visualized by staining with a monoclonal antibody against BrdU. Co-staining with an antibody against laminin served to identify the compartments where proliferation occurred. As expected, the wild-type cultures displayed areas of rapid mesenchymal proliferation around the tips of the ureter. The mesenchyme around the ureteric tips of the mutant cultures, however, showed very little BrdU incorporation when compared to the wild-type cultures, suggesting a lack of proliferation in the nephrogenic mesenchymal cells.

Discussion

Fgfrl1 is expressed in the kidney

Fgfrl1 is expressed in the tubule forming metanephric mesenchyme, with strongest expression observed in renal vesicles and the structures derived thereof. Previous data from in situ hybridization experiments and microarray analysis are in agreement with our immunohistochemical detection of Fgfrl1 in the metanephros. At E15.5 the Fgfrl1 protein is primarily found in the differentiating mesenchymal structures from the cortical zone of the growing kidney, while no signal is detected in the medullary stromal cells and in fully differentiated glomeruli. We observed strong focal staining of Fgfrl1 protein at sites of cell-cell contact in the epithelia of renal vesicles and further differentiated nephron precursors. This was not unexpected, as we have previously shown that the Fgfrl1 protein is often enriched at sites of cell–cell contact when overexpressed in mammalian cell lines, such as human embryonic kidney cells, rhabdomyosarcoma cells and osteosarcoma cells (Rieckmann et al., 2008; Trueb et al., 2003). The expression and localization of Fgfrl1 during tubulogenesis suggests that it might be involved in the differentiation and epithelialization of mesenchymal cells into nephron epithelium.

Indeed, the metanephric mesenchyme of our *Fgfrl1*-/- mice completely fails to undergo tubulogenesis, resulting in fully penetrant dysgenesis of both kidneys in the homozygous knock-out mice. Although the ureteric bud initially invades the metanephric mesenchyme and undergoes some rounds of branching, the mesenchyme appears to be largely unresponsive and nephron differentiation is not initiated. The rest of the urogenital system, including ureter, bladder and gonads, appears to be unaffected by the *Fgfrl1* deficiency. The drastic effect on the development of the metanephros is particularly intriguing as the mutant mice are otherwise well developed. As reported previously, our mice possess a hypoplastic diaphragm, which leads to postnatal respiratory failure (Baertschi et al., 2007), and display minor alterations in the bones of the skull and the limbs (Rieckmann et al., 2009). Given the strong expression in cartilage, tongue and diaphragm, the complete lack of the kidneys, which express lower amounts of *Fgfrl1* than those tissues, was not anticipated. The lung, another organ that undergoes branching morphogenesis and expresses *Fgfrl1* in differentiating tubules (www.genepaint.org), seems to be unaffected by the deficiency

Fig. 7. Whole-mount in situ hybridization of selected marker genes in E12.5 and E14.5 wild-type and *Fgfrl1* deficient kidneys. The tissues were hybridized with DIG-labeled RNA probes, followed by detection of the probe with alkaline phosphatase coupled antibodies. The ureteric marker genes *Wnt9b* and *Wnt11* are expressed both in the wild-type and in the mutant kidneys, as was calbindin used as a positive control. The tubulogenic marker genes *Fgf8*, *Wnt4*, *Pax8* and *Lim1* are expressed in the wild-type kidneys at both embryonic stages but could not be detected in the *Fgfrl1* deficient kidneys. *Six2*, a marker for the uninduced mesenchyme, is expressed in wild-type and mutant kidneys. The white arrows indicate the kidneys of the *Fgfrl1* knock-out embryos.

(Baertschi et al., 2007). It remains to be investigated in detail whether the lung and other tubule containing organs might show minor alterations in our *Fgfrl1* deficient animals.

Loss of Fgf8, Wnt4, Pax8 and Pax2 expression in Fgfrl1-/- kidneys

In order to understand the effects of *Fgfrl1* deficiency on tubulogenesis, we analyzed the expression of selected marker genes in E12.5 and E14.5 kidney rudiments. Although most of the genes analyzed were somewhat down-regulated in the mutant kidney rudiments, expression of the gene products that are characteristic for the ureteric bud, such as *Wnt9b*, *Wnt11* and the ureteric bud marker *Ehf*, was nearly intact. However, expression of those genes that drive nephron differentiation in the metanephric mesenchyme was dramatically reduced. At E12.5 and E14.5 the mRNA levels for *Wnt4*, *Fgf8*, *Pax8* and *Lim1* were reduced about tenfold and none of them could be detected by in situ hybridization, whereas they were readily detected in the wild-type kidneys.

In conditional knock-out mice, the tissue specific deletion of *Fgf8* and *Lim1* from the metanephric mesenchyme (Grieshammer et al., 2005; Kobayashi et al., 2005) resulted in a kidney phenotype similar to the one observed in our mice, although the developmental arrest of the *Fgfrl1* deficient kidneys appears to occur at an earlier stage than that found in the *Fgf8* and *Lim1* mutant mice. In both of the latter animals, nephrogenesis was initiated in the metanephric mesenchyme, but nephron development was arrested at the renal vesicle stage. In contrast, we did not find any renal vesicles in our mice. Wnt4 has been reported to be required for the initial mesenchymal-to-epithelial transition, as *Wnt4* mutant mice did not develop any nephron precursor structures such as renal vesicles (Stark et al., 1994). The absence of *Wnt4* expression in the *Fgfrl1*-/- kidney rudiments could therefore explain the lack of mesenchymal to epithelial conversion that we observed. The severely hypoplastic kidneys of the *Wnt4* mutant mice (Kobayashi et al., 2005; Stark et al., 1994) closely resemble the rudimentary kidney structures that we found in the *Fgfrl1*-/- mice.

Failure of mesenchymal induction

Taken together, our data suggest that the uninduced *Fgfrl1* deficient metanephric mesenchyme, as specified by the nearly intact expression of *Pax2*, *Wt1*, *Eya1* and *Six2* (Carroll et al., 2005), was not only present, but was also clearly capable of inducing budding, invasion and some branching of the ureteric bud. During this invasion and branching process, the epithelial cells of the ureter produce and secrete Wnt9b, which induces *Fgf8*, *Pax8* and *Wnt4* and subsequently *Lim1* expression in the surrounding mesenchymal cells in a paracrine fashion (Carroll et al., 2005). Our real-time PCR results and the in situ hybridizations indicate that *Wnt9b* was still expressed by the *Fgfrl1* deficient ureteric epithelium, while *Fgf8*, *Pax8*, *Wnt4* and *Lim1* expression was not induced in the mesenchyme. Apparently, the mesenchyme remained in its uninduced state, failed to express these critical tubulogenic markers and consequently did not initiate nephrogenesis.

The defect in mesenchymal nephrogenesis was accompanied by an arrest of ureteric branching, which is similar to the abrupt arrest of ureteric outgrowth observed in *Wnt4* and *Wnt9b* deficient animals (Stark et al., 1994; Carroll et al., 2005). We assume that this is a secondary defect due to a loss of reciprocal inductive signals from the mutant mesenchyme. Our real-time PCR data indicated that *Gdnf* and especially *c-Ret* expression were reduced at E14.5. Since Gdnf and its receptor c-Ret are critical for the initial budding of the ureter and for the maintenance of ureteric branching morphogenesis (Dressler, 2006), it is possible that this reduction ultimately led to the arrest of ureteric outgrowth.

Most likely as a consequence of a prolonged lack of induction and of missing survival signals from differentiating nephrons, we also detected a loss of Pax2 positive nephrogenic precursor cells and an increase of apoptotic cells in the cortical zone of the remnant kidney at E14.5. The observation that the pattern of apoptotic cells resembles the aberrant cell death found in kidneys of mice deficient in *Fgf8* mediated survival signals (Grieshammer et al., 2005) is in agreement with this hypothesis. The decrease of mesenchymal proliferation detected in our mutant organ cultures together with the increase in cell death in our knock-out animals might explain why only a small number of Pax2 positive nephron precursor cells was left around the ureteric tips of the mutant kidney at E14.5.

With regard to the molecular events that underlie the failure of mesenchymal induction and tubulogenesis in the *Fgfrl1*-/- kidneys, we can only speculate. Several Fgf ligands and receptors have been shown to be important during early kidney development. Compound deletion of *Fgfr1* and *Fgfr2* from the metanephric mesenchyme resulted in renal aplasia due to a loss of proper mesenchyme formation (Poladia et al., 2006). We have shown that Fgfrl1 binds Fgf2 in vitro (Trueb et al., 2003) and that it can interfere with Fgf2 signaling in cultured cells (Rieckmann et al., 2009). It is therefore possible that Fgfrl1 modulates Fgfr1 and/or Fgfr2 signaling in the metanephric mesenchyme in a critical way. During developmental processes, Fgf signaling often acts in concert with Wnt-mediated signaling to evoke the full-blown response (Katoh and Katoh, 2006). Fgf8 for example is needed to initiate *Wnt4* expression in the pretubular aggregates of the metanephric mesenchyme and, in turn, Wnt4 signaling reinforces *Fgf8* expression and signaling in the nascent renal vesicle (Grieshammer et al., 2005; Schedl, 2007). With regard to the Wnt9b mediated induction of the metanephric nephron precursor cells, no such concomitant Fgf pathway has been described so far. Given the strikingly similar kidney phenotypes of *Wnt9b* deficient mice (Carroll et al., 2005) and *Fgfrl1* deficient mice, one is tempted to speculate that Fgfrl1 might mediate or modulate an Fgf signal that acts in concert with Wnt9b to initiate the tubulogenic program in nephron precursor cells.

An alternative explanation could lie in the fact that Fgfrl1 displays similarities with other Ig-domain superfamily members that are involved in cell adhesion and cell-cell recognition. We have shown that Fgfrl1 forms constitutive dimers that are enriched at sites of cell-cell contact and that promote cell adhesion (Rieckmann et al., 2008). In light of this observation, it is conceivable that Fgfrl1 is required to establish cell–cell contacts and thereby cell identity in pretubular aggregates and structures derived thereof. The dramatic effect of *Fgfrl1* deficiency on nephrogenesis and the widespread expression of *Fgfrl1* in other developing tissues certainly warrant further investigation into this novel receptor molecule.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.08.019.

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